Bovine γ/δ T Cells Bind E-Selectin via a Novel Glycoprotein Receptor: First Characterization of a Lymphocyte/E-Selectin Interaction in an Animal Model

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Summary

E-Selectin is an inducible adhesion protein expressed by endothelial cells and recognized by leukocytes during their extravasation from the blood into inflamed tissues. Originally, E-selectin was defined as a myeloid cell–specific adhesion protein, but recent studies have shown it to be recognized by human lymphocytes as well. These lymphocytes represent a memory T cell subset and have been shown to express the HECA-452 carbohydrate epitope (CLA^+ lymphocytes). We extend these findings and show that ruminant γ/δ T cells bind E-selectin as well; and we provide preliminary evidence that this interaction is mediated by a novel glycoprotein receptor on the lymphocyte. Unlike conventional T cells (α/β T cells), γ/δ T cells from neonatal and mature animals bind E-selectin, suggesting that prior antigen stimulation and differentiation to a memory lymphocyte are not required for this interaction. Neuraminidase treatment of the γ/δ T cells or addition of ethylenediaminetetraacetic acid (EDTA) to the assay abrogates binding, demonstrating the importance of sialic acid and divalent cations, which is consistent with other E-selectin–mediated adhesion events. However, previously defined E-selectin carbohydrate ligands, such as sialyl Lewis x on neutrophils and the HECA-452 epitope on human memory lymphocytes, are antigenically different than the carbohydrates on ruminant γ/δ T cells since the mAbs CSLEX and HECA-452 do not recognize these cells. Protease treatment of γ/δ T cells significantly inhibits their binding to E-selectin; however, previously characterized adhesion glycoproteins, such as L-selectin, CD44, and CD18, are not involved in the adhesive event. An E-selectin affinity column purifies a single glycoprotein of 250 kD (280 kD under reducing conditions) from γ/δ T cell detergent lysates. Neuraminidase digestion of the 250-kD product as well as EDTA abolishes binding to E-selectin. Finally, E-selectin expression in vivo appears to mediate γ/δ T cell accumulation. Stimulation of bovine skin with tumor necrosis factor α induced an increase in E-selectin expression that was associated with an influx of γ/δ T cells at the same site.

Selectins are a new family of adhesion proteins that direct leukocyte entry into sites of inflammation and lymphoid tissue (1). Two vascular (P- and E-selectin) and one leukocyte (L-selectin) selectin have been defined. From the predicted cDNA sequences, selectins are composed of an NH_2-terminal, C-type lectin domain followed by an epidermal growth factor domain, and multiple short consensus repeats (SCR)[1] that are homologous to complement-binding proteins. Selectins also have conventional transmembrane sequences and short cytoplasmic tails. The selectin lectin domains are required for function and their carbohydrate-binding specificities have been partially defined. Sialylated Lewis carbohydrate derivatives, such as sialyl Lewis x (sLex) and sialyl Lewis a (sLea), have been shown to function as selectin ligands (2–4).

E-Selectin was originally described as a myeloid cell–specific adhesion protein whose expression on cytokine-activated endothelial cells in vivo was thought to direct the entry of neutrophils and monocytes into sites of acute inflammation (5). However, E-selectin has been found on venules in sites of chronic inflammation that are predominantly associated with infiltrates of mononuclear cells (6, 7). Recently, human CD4 memory T cells, but not virgin cells, have been shown to avidly bind E-selectin (8, 9). The capacity to bind E-selectin after the acquisition of the memory phenotype is associated with the expression of the HECA-452 epitope (CLA antigen) on T cells (8, 10, 11). HECA-452 recognizes the "face" of the carbohydrate moiety on lymphocytes that is bound by

[1] *Abbreviations used in this paper:* HEV, high endothelial cell venule; SCR, short consensus repeat; sLea, sialyl Lewis a; sLex, sialyl Lewis x; WC1, Workshop Cluster 1.

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E-selectin (12). It has been suggested that the interaction between memory T cells and E-selectin may be important in the preferential accumulation of these lymphocytes in certain inflammatory sites (8–10). Picker et al. (8, 10) have extended this hypothesis, based on their observation that E-selectin appears to be preferentially expressed on venules in the skin, and proposed that E-selectin serves as a vascular addressin for lymphocyte recirculation through dermal tissue. The testing of these hypotheses has not been done due to the fact that all information obtained thus far has been with human cells and not with cells from animal models.

In many animals, including humans, γ/δ T cells are thought to have the capacity to recirculate through epithelial cell–associated tissues (13, 14), such as gut and skin, versus a preference of conventional T cells (α/β) for secondary lymphoid organs (15). In particular, γ/δ T cells in ruminants exhibit a considerable capacity to home to the skin (14). Since E-selectin may be a skin-specific addressin, we tested if ruminant γ/δ T cells represent another lymphocyte subset that binds E-selectin. If true, the role of these interactions in vivo could eventually be tested.

We show that bovine γ/δ T cells avidly bind human E-selectin–transfected L cells. Unlike human CD4 T cells, prior differentiation or acquisition of the HECA-452 epitope is not required for binding. In addition, neuraminidase or protease treatment of the γ/δ T cells blocks adhesion, but known adhesion proteins, such as CD18, CD44, and, importantly, L-selectin, appear to play no role in binding E-selectin. A single 250-kD glycoprotein, isolated from detergent lysates of γ/δ T cells using an E-selectin affinity column, may serve as the E-selectin receptor on the lymphocyte. The interaction defined here appears important in vivo, since upregulation of E-selectin in bovine skin by injection of TNF-α increases the accumulation of γ/δ T cells at the same site.

These results provide the first demonstration that lymphocytes in an animal model bind E-selectin. Thus, the role of this interaction in vivo can be tested. In addition, the γ/δ T cell's receptor for E-selectin may represent a member of a potentially new family of highly glycosylated surface proteins that serve as high affinity ligands for selectins.

Materials and Methods

Animals. Mixed-breed cattle, ranging in age from 1 to 4 wk, were purchased from local producers and housed at the Montana State University large animal facilities at the Veterinary Molecular Biology Laboratory. Human and bovine peripheral blood was collected into heparinized tubes by venipuncture, and the PBMC were separated by centrifugation through Ficoll-Hypaque (histopaque 1077; Sigma Chemical Co., St. Louis, MO). Bovine tissue samples were collected from the animals upon necropsy.

Reagents. The mAbs used in this report have been described elsewhere. DREG-56 is a mouse mAb that recognizes human (16) and bovine (17) L-selectin. IL-A29 (American Type Culture Collection, Rockville, MD) and BAQ4A (Veterinary Medical Research and Development, Pullman, WA) are mouse mAbs that recognize the Workshop Cluster 1 (WC1) antigens (also referred to as T19) specifically expressed by bovine, ovine, and caprine γ/δ T lymphocytes (18–22). Hermes 3 (23–25) is a mouse mAb that recognizes CD44 (HCAM) on human leukocytes and crossreacts with bovine lymphocytes (17). 60.3 (generously provided by John Harlan, University of Washington, Seattle, WA) is a mouse mAb that recognizes CD18 on human leukocytes (26) and crossreacts with ruminant leukocytes. EL-246 (27) and CL2 (28) are mouse mAbs that recognize the SCR and lectin domains of human E-selectin, respectively. EL-246 also crossreacts with bovine L- (27) and E-selectin (M. A. Jutila, personal observation). EL-81 is a mouse mAb that recognizes a nonfunction-blocking epitope on human E-selectin (27). CSLEX is a mouse mAb that recognizes the Lewis carbohydrate derivative sLex (29), and it also crossreacts in the ruminant (M. A. Jutila, personal observation). HECA-452 is a rat mAb that recognizes a carbohydrate moiety expressed on high endothelial cell venules (HEV) (30), myeloid cells (11), and the CLA antigen (12) expressed on a subset of human memory T cells. HECA-452 also crossreacts with lymphoid tissue postcapillary venules in the ruminant (M. A. Jutila, personal observation).

In Vitro Binding Assay. In all binding assays, human E-selectin–transfected L cells (E-selectin–L cells) (generously provided by T. K. Kishimoto, Boehringer Ingelheim, Ridgefield) or the parent cell line control were grown on eight-well Labtek slides (Miles Scientific, Naperville, IL) as previously described (27). Approximately 5×10^5 PBMC, resuspended in 400 μl HB101 (Irvine Scientific, Santa Ana, CA) serum-free media, were allowed to adhere to the transfectants at room temperature for 30 min under constant rotation; the slides were then washed in HBSS (JRH Biosciences, Lenexa, KS) and fixed in 1% glutaraldehyde (Fisher Scientific, Fair Lawn, NJ). All mAbs used in the blocking assays were at a final concentration of 50 μg/ml. Neuraminidase (Sigma Chemical Co.) treatment (1 U/ml in 50 mM NaOAc, pH 5.5, 154 mM NaCl, 4 mM $CaCl_2$) of the PBMC was performed at 37°C for 30 min. Similarly, PBMC were treated with chymotrypsin and trypsin (Sigma Chemical Co.) (100 U/ml in HBSS) for 60 min at 37°C. For the specific removal of leukocyte L-selectin, PBMC were treated with low-dose chymotrypsin (0.5 U/ml) or the chemical crosslinker BS^3 (Pierce Chemical Co., Rockford, IL), as previously described (17, 31, 32). After the various treatments, the PBMC were washed in HBSS, and the binding assay was performed as described above. All control experiments were performed under the same conditions as the treated cells, but without addition of the enzyme. After the binding assay, γ/δ T cells were revealed by immunoperoxidase staining using a biotin-labeled IL-A29 mAb, streptavidin peroxidase (Tago, Inc., Burlingame, CA), and amino-ethyl carbazol (Sigma Chemical Co.).

Immunofluorescence Staining and FACS® Analysis. The immunofluorescence staining procedure was carried out in 4-ml tubes (Becton Dickinson & Co., Mountain View, CA). Typically, 10^6 cells per tube were initially incubated in 2% rabbit serum for 10 min on ice to block Fc receptors. The cells were washed and then incubated with primary antibody at 50 μg/ml (or undiluted culture supernatant) for 20 min on ice. After washing, bound antibodies were revealed by incubation with PE-conjugated $F(ab')_2$ goat anti-mouse Ig (Tago, Inc.). FACS® analysis was performed on a FACScan® (Becton Dickinson & Co.) as previously described (31–34). In all analyses, lymphocytes were identified by their unique forward and side light scatters. For two-color analysis, FITC-conjugated IL-A29 was used after staining with the primary mAb. Before cells were stained with the FITC-conjugated mAb, they were treated with 10% mouse serum to block any available anti-mouse Ig binding sites of the second stage. Data were collected from 10,000 cells and are presented as contour plots.

EL-81-E-Selectin Sepharose 4B Column Preparation. The nonfunction-blocking, anti-E-selectin mAb EL-81 (5 mg) was coupled

to Sepharose 4B beads (Pharmacia LKB, Uppsala, Sweden) (1.5 ml swollen bead volume) per manufacturer's instructions. Approximately 3×10^8 E-selectin–L cells were lysed in 2% NP-40 (Sigma Chemical Co.), 0.1 M NaCl (Sigma Chemical Co.), 10 mM Hepes (Mediatech, Washington, DC), 1 mM $CaCl_2$ (Sigma Chemical Co.), 1 mM $MgCl_2$ (Sigma Chemical Co.), 5 mM NaN_3 (Sigma Chemical Co.), with 10 μg each of pepstatin, leupeptin, chymostatin, antipain, benzamidine HCl, 1, 10 phenanthroline, and 1 mM PMSF (Sigma Chemical Co.), and then centrifuged at 10,000 *g* for 15 min. The lysate was incubated on the EL-81 column for 24 h at 4°C. The EL-81–E-selectin column was then eluted with 0.1% NP-40, 0.1 M NaCl, 10 mM Hepes, 5 mM EDTA (Sigma Chemical Co.), 5 mM NaN_3 and 1 mM PMSF to remove residual binding proteins left from the E-selectin–L cell lysate, and extensively washed with 0.1% NP-40, 0.1 M NaCl, 10 mM Hepes, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM NaN_3, and 1 mM PMSF.

Purification of the γ/δ T Cell E-Selectin Receptor. Bovine PBMC were collected, adherent cells removed by incubation in a polystyrene flask at 37°C plus 10% CO_2 for 1 h, and the nonadherent cells (5×10^8) lysed as described above for the E-selectin–L cells. Equivalent numbers of a mouse pre-B tumor cell line (L1.2) or a nonhematopoietic tumor cell line (L cells) were lysed and used as controls for the E-selectin affinity column procedure. Lysates were precleared over an EL-81 column (no E-selectin) for 24 h at 4°C. The precleared filtrate was collected, added to the EL-81–E-selectin column, and incubated for 24 h at 4°C. The EL-81–E-selectin column filtrate was collected, the column rinsed extensively with wash buffer, and eluted with 5 mM EDTA elution buffer. Samples were solubilized in 6× Laemmli's sample buffer, subjected to electrophoresis through an 8% SDS-polyacrylamide gel under reducing or nonreducing conditions, and detected by silver stain analysis (Bio-Rad Laboratories, Richmond, CA) per manufacturer's instruction.

Neuraminidase Digestion of Purified Protein. Protein purified from the EL-81–E-selectin column was neuraminidase digested, similar to the conditions described by Moore et al. (35) for optimal sialic acid removal. Protein-containing EDTA elution fractions were consolidated and the buffer exchanged into a neuraminidase reaction buffer (0.15 M NaCl, 50 mM acetate, pH 6.0, 9 mM $CaCl_2$, 0.005 mM NaN_3, 0.005% NP-40) by use of a Centricon 30 concentrator (Amicon Corp., Beverly, MA). The digestion was performed at 37°C for 10 h in the presence or absence of 500 mU/ml neuraminidase from *Arthrobacter ureafaciens* (Calbiochem-Behring Corp., La Jolla, CA).

TNF-α Stimulation of Bovine Skin. Approximately 5–10 μg of mouse TNF-α (Genentech, San Francisco, CA) in 200 μl sterile HBSS was injected intradermally to raise a diffuse bleb. Tissue from stimulated and unstimulated (contralateral) regions were collected and frozen in optimal cutting temperature compound (Miles Inc., Elkhart, IN). 6-μm skin sections were cut, fixed in acetone, and blocked with 10–15% rabbit serum for 20 min. γ/δ or α/β T cells were revealed by immunoperoxidase staining using the IL-A29 or CC42 mAbs, respectively. E-Selectin expression was detected by the EL-246 mAb. The primary mAbs were revealed by second-stage biotinylated anti–mouse antibody (Tago, Inc.); streptavidin peroxidase, and amino-ethyl carbazol. Tissue sections were also incubated with isotype-matched negative control mAbs to determine any nonspecific binding of antibodies and/or endogenous peroxidase development of the substrates.

Results

Bovine γ/δ T Cells Bind E-Selectin cDNA-transfected Mouse L Cells. Isolated bovine mononuclear cells were tested for their ability to bind mouse L cells transfected with human E-selectin cDNA (E-selectin–L cells). The binding assay was performed under rotation at room temperature. After the assay and fixation of bound cells, the γ/δ T lymphocytes were revealed by immunoperoxidase staining using a lineage-specific, anti-WC1 mAb. As shown in Fig. 1 *A*, bovine γ/δ T cells avidly bound the E-selectin–L cells, but not the nontransfected parent cell line control (Fig. 1 *B*). Immunoperoxidase analysis demonstrated that γ/δ T cells represented >90% of the mononuclear cells, when isolated from neonatal animals, that bound to the transfectants. Monocytes and, perhaps, memory T cells made up essentially the rest of the binding population. Like their human counterparts (5), bovine neutrophils also bound the E-selectin transfectants (data not shown).

To address what percentage of the γ/δ T cell population has the capacity to bind E-selectin, a mixed PBMC suspension was sequentially passed over different monolayers of the E-selectin transfectants. Greater than 90% of the γ/δ T cells bound E-selectin and were removed from the PBMC population (data not shown).

Characterization of the Binding Interaction between γ/δ T Cells and E-Selectin. We initially tested the effects of anti–E-selectin mAbs on the binding of γ/δ T cells to E-selectin. EL-246 and CL2, which recognize the SCR and lectin domains of E-selectin (27, 28), respectively, effectively blocked the binding interaction (Table 1). EL-81, which is a nonblocking, anti–E-selectin mAb (27), had little effect (Table 1). Neuraminidase treatment of the γ/δ T cells or addition of 5 mM EDTA to the binding assay also abrogated adhesion (Table 1). These results demonstrate that E-selectin supports the adhesion of γ/δ T cells and that the binding interaction is dependent upon divalent cations. Furthermore, the apparent affinity necessary for γ/δ T cells to adhere to the E-selectin–L cells during the rotational binding assay requires sialylated carbohydrates on the lymphocyte's cell surface. These characteristics are similar to other previously defined leukocyte/E-selectin interactions (2, 36–40).

Next we tested if bovine γ/δ T cells are recognized by mAbs that bind carbohydrates that support E-selectin adhesion in other systems, such as CSLEX and HECA-452 (8, 10, 36–40). In other studies we have found that HECA-452 crossreacts in the ruminant and stains peripheral lymph node postcapillary venules (M. A. Jutila, unpublished observations), which is similar to its reactivity in the human (30). However, we found that HECA-452 did not recognize bovine PBMC (Fig. 2 *a*). The CSLEX mAb also crossreacted in the cow, but stained <5% of bovine γ/δ T cells (Fig. 2 *b*). The lack of staining of bovine γ/δ T cells was not due to poor antibody preparations, since the HECA-452 and CSLEX mAbs did recognize human PBMC (Fig. 2, *c* and *d*, respectively). These results suggest that the sialylated carbohydrate structures recognized by E-selectin on γ/δ T cells are antigenically different than previously characterized E-selectin carbohydrate ligands.

In other systems, glycolipids and glycoproteins have been shown to present E-selectin carbohydrate ligands (41, 42).

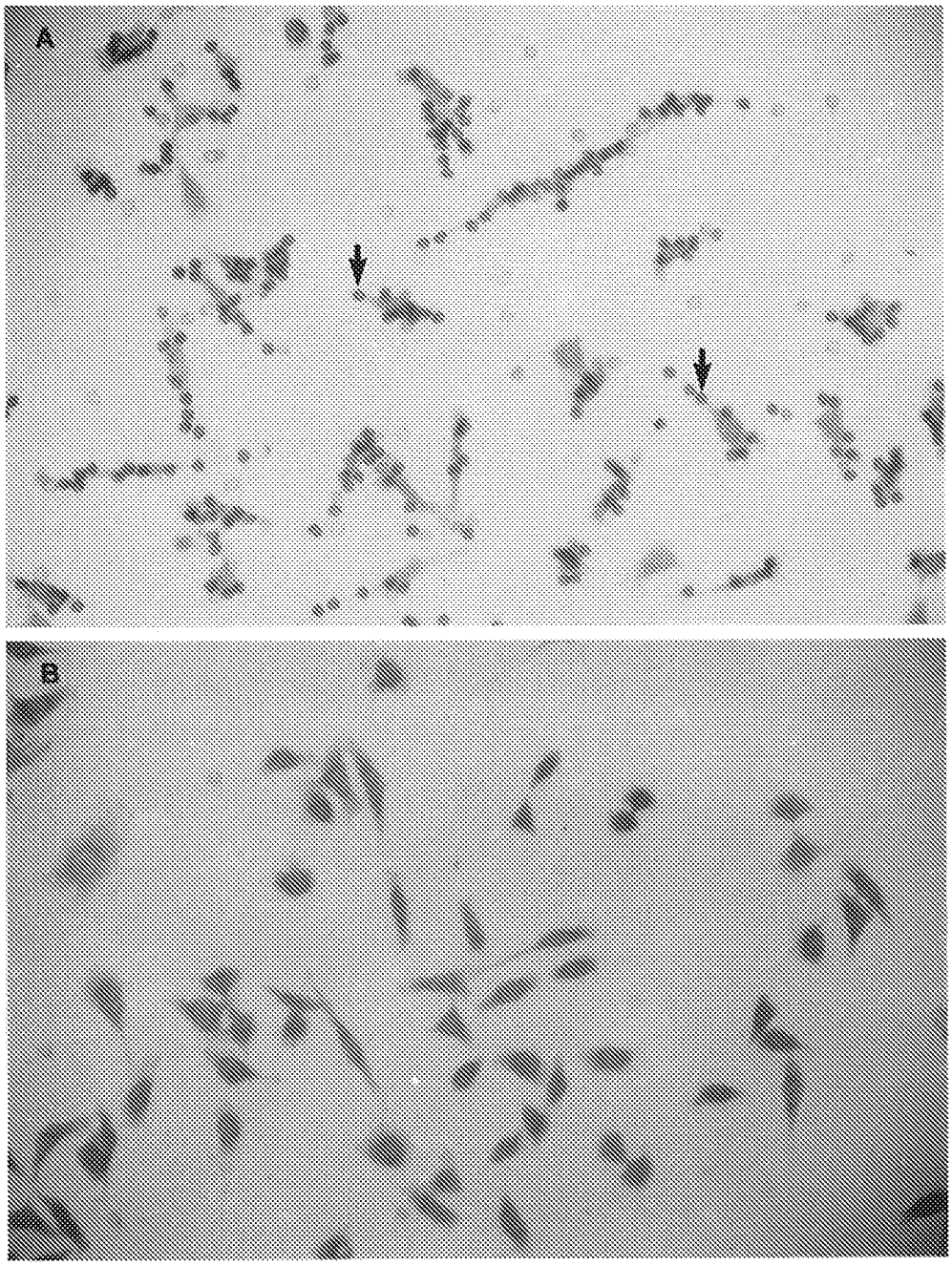


Figure 1. Bovine γ/δ T cells bind E-selectin–transfected L cells. Bovine PBMC, isolated from neonatal animals, were incubated with the E-selectin–L cells (*A*) or the parent cell line control (*B*) under nonstatic conditions at room temperature as described in Materials and Methods. The γ/δ T cells were revealed by immunoperoxidase staining using the mAb IL-A29 (*arrow*), which recognizes the lineage-specific WC1 antigen. Approximate magnification is 200.

To address whether protein or lipid is important to the γ/δ T cell presentation of E-selectin ligands, the effects of different proteases on the binding interaction were tested. Table 2 shows that chymotrypsin or trypsin treatment of bovine PBMC blocked binding by an average of 72 and 35%, respectively. Due to the protease sensitivity of the binding interaction, we examined the effects of three function-blocking antibodies against known glycoprotein adhesion molecules on γ/δ T cells (L-selectin, CD18, and CD44) for an inhibitory effect on E-selectin binding. Table 2 shows that none of these antibodies effectively blocked binding. Because L-selectin on neutrophils has been been proposed to be a ligand for E-selectin in the human (28, 42), and since γ/δ T cells express three to five times the level of this receptor as other leukocytes (Walcheck, B., manuscript in preparation), we examined the effects of other treatments that block L-selectin function. BS[3] or low-dose chymotrypsin (0.5 U/ml) treatments reduced the expression of L-selectin on the γ/δ T cells by >95% (data not shown), which is the same result as seen with other leukocytes (17, 31, 32, 42); but these agents had no significant effect on the binding interaction (Table 2). Our results demonstrate that both sialylated carbohydrates and a protein com-

Table 1. *Characterization of the Binding Interaction between γ/δ T Cells and E-Selectin*

Treatment of:	No. of γ/δ T cells/L cell* Treated	Control	Percent inhibition of binding‡
E-selectin–L cells§			
EL-246	0.9 ± 0.1	4.5 ± 1.0	79 ± 3.5
CL2	1.6 ± 0.3	4.5 ± 1.0	64 ± 1.1
EL-81	4.8 ± 1.0	4.5 ± 1.0	ND
EDTA	0	5.1 ± 0.5	100
γ/δ T cells‖			
Neuraminidase	0.1 ± 0.1	2.8 ± 1.6	98 ± 0.5

* γ/δ T cell binding to the E-selectin–L cells was quantified by counting three separate fields at 20× power. The mean number of γ/δ T cells per E-selectin–L cell was determined for control and treated cells. The values given represent the mean from three separate experiments ± SE.
‡ Percent inhibition of binding = 100× (control mean − treated mean/control mean). The values given represent the mean inhibition from three separate experiments ± SE.
§ E-selectin–transfected L cells were pretreated with the anti–E-selectin mAbs EL-246, CL2, or EL-81 at 50 μg/ml for 30 min, and the binding assay was performed as described in Materials and Methods. 5 mM EDTA was added to the E-selectin–L cells before the addition of the PBMC. γ/δ T cells, bound to the E-selectin–L cells after the binding assay, were revealed by immunoperoxidase staining with the lineage-specific mAb biotin-conjugated IL-A29.
‖ PBMC were treated with neuraminidase (1 U/ml) before their addition to the E-selectin–L cells.

ponent are important for γ/δ T cell binding to E-selectin, but that certain known adhesion molecules are likely not involved (further evidence provided below).

E-Selectin Binds a Single Glycoprotein in Detergent Lysates of γ/δ T Cells. Purified E-selectin was immobilized on a supporting matrix to isolate putative receptors from a detergent lysate of γ/δ T cells. Sepharose 4B beads coupled to a nonfunction-blocking anti–E-selectin antibody (EL-81) were used to capture detergent-solubilized E-selectin derived from the E-selectin–L cells. γ/δ T cells were lysed in NP-40 to isolate functional cell surface membrane complexes; precleared on an EL-81 column, which removed a considerable amount

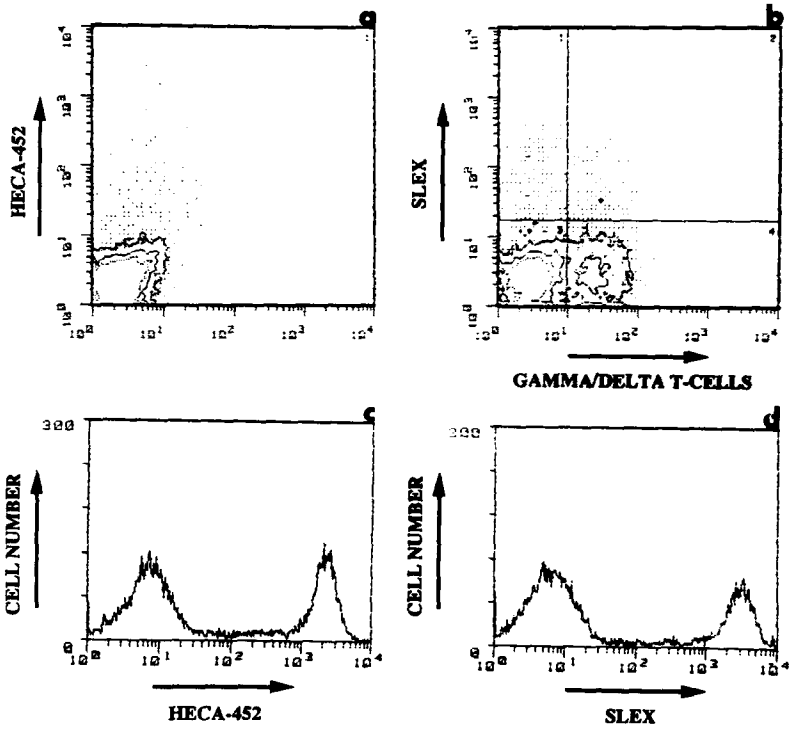
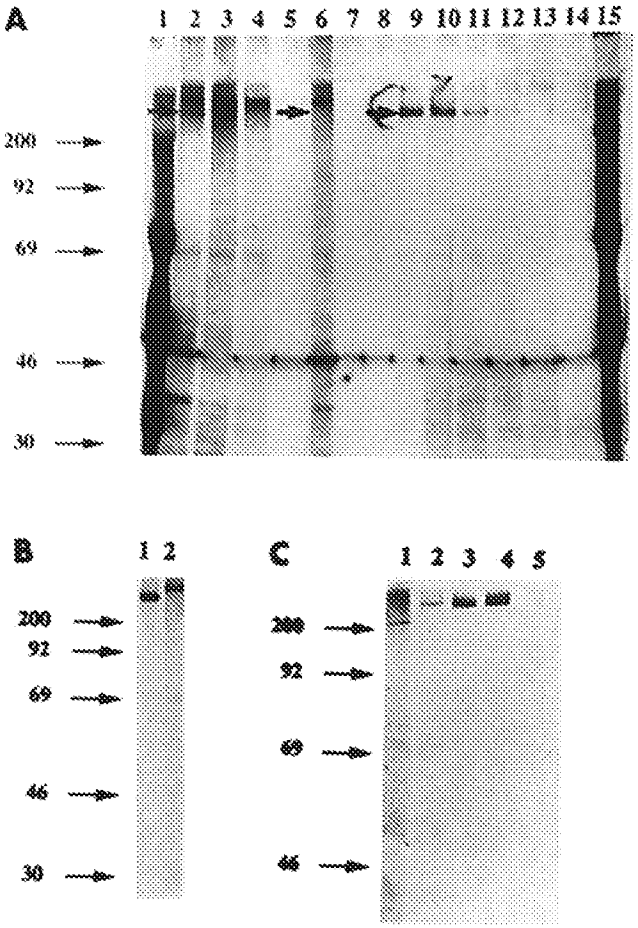


Figure 2. Bovine PBMC lack significant expression of the sLex and HECA-452 carbohydrate moieties. PBMC were separated and stained for FACS® analysis by the mAb HECA-452. For two-color FACS® analysis, the γ/δ T cells were stained by the mAbs FITC-conjugated IL-A29 and CSLEX as described in Materials and Methods. (*a*) Level of HECA-452 expression on PBMC, which is equivalent to the level of staining demonstrated by an isotype-matched negative control antibody. (*b*) Level of sLex expression on γ/δ and non-γ/δ T cells. Less than 5% of the γ/δ T cells expressed sLex after background fluorescence with an isotype control mAb was subtracted out of the percentage of positive staining cells. (*c* and *d*) sLex and HECA-452 antigen expression on human PBMC, respectively.

Table 2. *The Effects of Function-blocking mAbs against γ/δ T Cell Adhesion Glycoproteins on E-Selectin Binding*

Treatment of γ/δ T cells	Adhesion protein	No. of γ/δ T cells/L cell*		Percent inhibition of binding‡
		Treated	Control	
Hermes-3§	CD44	3.4 ± 0.6	3.5 ± 0.2	ND
60.3	CD18	3.5 ± 0.5	3.5 ± 0.2	ND
DREG-56	L-Selectin	4.3 ± 0.3	3.5 ± 0.2	ND
Chymotrypsin (0.5 U/ml)‖	L-Selectin	1.9 ± 0.5	2.2 ± 0.3	ND
BS^3	L-Selectin	2.0 ± 0.6	2.2 ± 0.3	ND
Chymotrypsin (100 U/ml)¶		1.0 ± 0.4	4.6 ± 0.7	72 ± 9.3
Trypsin (100 U/ml)		3.2 ± 1.1	4.6 ± 0.7	35 ± 11

* γ/δ T cell binding to the E-selectin–L cells was quantified as described in Table 1.
‡ Percent inhibition of binding = 100× (control mean − treated mean/control mean). The values given represent the mean inhibition from three separate experiments ± SE.
§ γ/δ T cells were pretreated with the mAbs Hermes-3, 60.3, or DREG-56 at 50 μg/ml for 30 min, and the binding assay was performed as described in Table 1.
‖ γ/δ T cell L-selectin was removed from the cell surface by treatment of these cells with low-dose chymotrypsin or the chemical crosslinker BS^3 as described in Materials and Methods.
¶ γ/δ T cell surface membrane proteins were removed by protease treatment with chymotrypsin or trypsin as described in Materials and Methods.



of protein; and then incubated on the EL-81–E-selectin column. Material bound to both the preclearing and E-selectin columns was eluted using a 5-mM EDTA elution buffer. As shown in Fig. 3 *A*, a predominant molecule of ~250 kD was extracted by the EL-81–E-selectin column and detected in the elution fractions 3–6 (lanes *9–12*) by nonreducing SDS-PAGE and silver stain analysis. The 250-kD band was faintly

Figure 3. Silver stain analysis of a single γ/δ T cell glycoprotein isolated from an E-selectin affinity column. (*A*) Samples from the respective steps involved in the purification of the E-selectin ligand from a γ/δ T cell lysate were subjected to electrophoresis on 8% SDS-polyacrylamide gels under nonreducing conditions and revealed by silver stain analysis as described in Materials and Methods. Lanes *2* and *3* represent the filtrates from a γ/δ T cell lysate sequentially passed over the EL-81 preclearing column twice (lane *2*, first passage; lane *3*, second passage [*arrow*, presence of the 250-kD band]). Lane *4* represents a sample of the consolidated EDTA elution fractions 3, 4, and 5 from the EL-81 preclearing column. Lane *5* represents 6× Laemmli's sample buffer plus running buffer. Lane *6* represents the filtrate of the precleared γ/δ T cell lysate collected from the EL-81–E-selectin column (*arrow*, absence of the 250-kD band). Lanes *7–14* represent the EDTA elution fractions 1–8, respectively, from the EL-81–E-selectin column (*arrow*, presence of the 250-kD band). Lanes *1* and *15* represent molecular mass markers, which are designated on the left of the figure by their respective mass. (*B*) The 250-kD product was run on an 8% polyacrylamide gel under nonreducing (lane *1*) and reducing (lane *2*) conditions. (*C*) Equal concentrations of the 250-kD product were neuraminidase digested or sham treated, then reisolated by the E-selectin affinity column (lanes *5* and *4*, respectively). An equivalent number of a mouse pre-B (L1.2 cells) or nonhematopoietic (L cells) tumor cell line to γ/δ T cells were NP-40 solubilized and affinity column extracted as described above. EDTA elution fractions from the EL-81–E-selectin column from each lysate were consolidated and equal sample volumes analyzed; lane *1*, L cells; lane *2*, L1.2 cells; lane *3*, γ/δ T cells.

evident in the filtrate from the preclearing column, but undetected in the EDTA elution fractions from the same column. The 250-kD was not detected in the E-selectin column filtrate (Fig. 3 *A*); however, all other bands seen in the filtrate off the preclearing column were still present (compare lanes *2* and *6*). Some minor protein bands were present in the EL-81–E-selectin elution fractions as well. For instance, a molecule of 69 kD was detected; however, this molecule was also evident in the elution fractions off the preclearing column and the filtrate from the E-selectin column (Fig. 3 *A*). Thus, we believe it interacted with EL-81 and/or the supporting beads, but not E-selectin. A continuous background band was also seen at ~46 kD, which was generated by a contaminating protein within the Laemmli's sample buffer. The E-selectin affinity-purified molecule migrated at ~280 kD under reducing conditions (Fig. 3 *B*), which consisted of solubilization at 37°C with 2-ME due to the 250-kD molecule's sensitivity to boiling (it may aggregate).

As established above, the binding interaction between γ/δ T cells and E-selectin is dependent upon sialic acid. Related studies by Moore et al. (35) describing a myeloid glycoprotein receptor for P-selectin have demonstrated the importance of sialic acid to the function of their molecule as well. Using the same neuraminidase enzyme, buffer, and incubation conditions determined by Moore et al. to be optimal for the removal of sialic acid residues from their glycoprotein, we digested the purified 250-kD molecule to determine if it could be reisolated by the E-selectin affinity column. As shown in Fig. 3 *C*, reactivity of the 250-kD molecule with E-selectin was greatly diminished by prior neuraminidase digestion, though treatment of an equal concentration of the molecule under the same conditions without neuraminidase had no apparent effect on the binding interaction. Neuraminidase digestion of the 250-kD molecule decreased its mobility during SDS-PAGE (equivalent to 300 kD; data not shown), which is characteristic of heavily sialylated glycoproteins (43, 44)

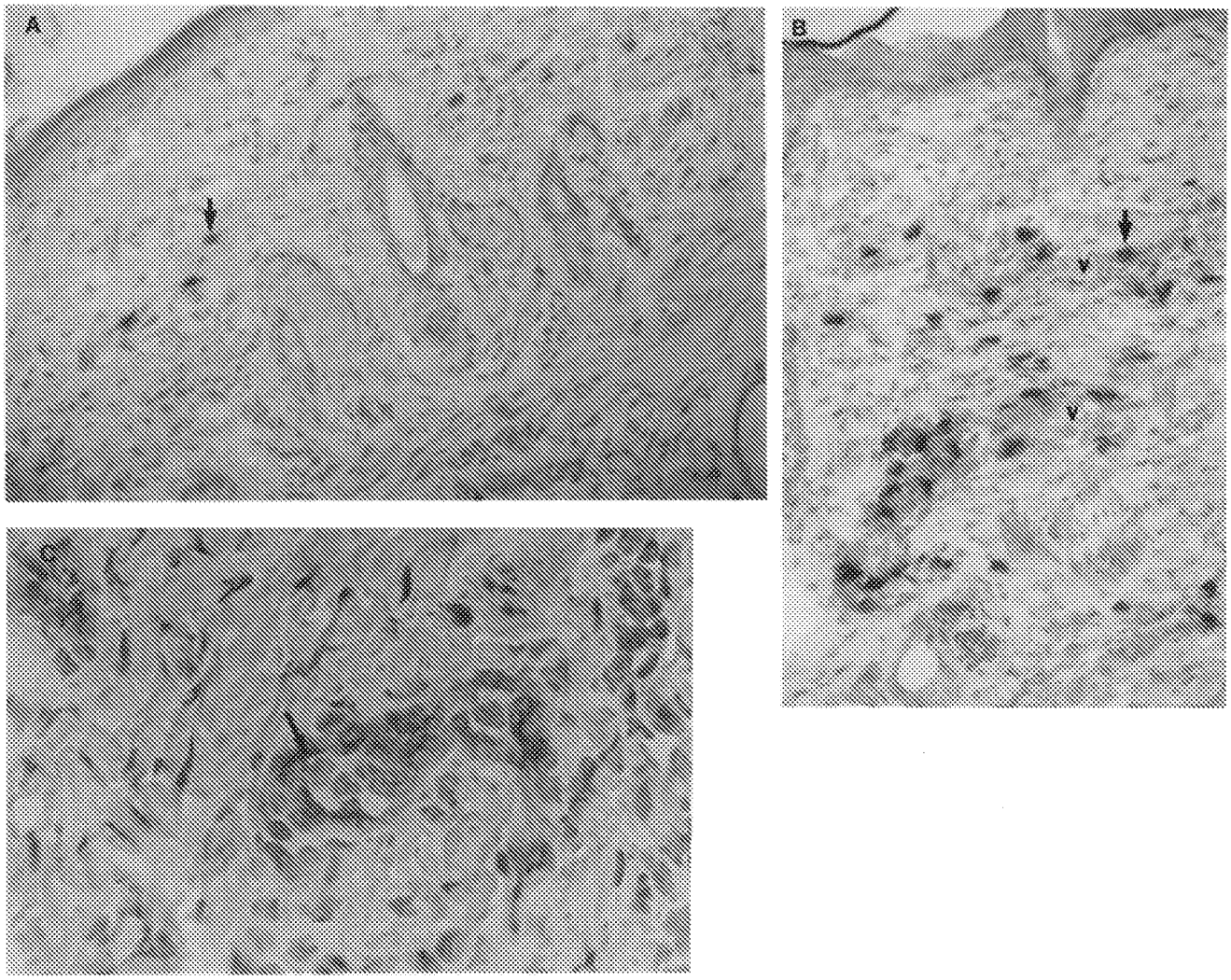


Figure 4. TNF-α-induced, endothelial cell E-selectin expression in skin is associated with an accumulation of γ/δ T cells at the same site. Bovine skin was stimulated for 6 h by intradermal injection of 10 μg TNF-α. Sections cut from control (*A*) and stimulated skin (*B*) samples were stained by immunoperoxidase using the γ/δ T cell–specific mAb IL-A29 (*arrow*; *v*, venule). (*C*) TNF-α-stimulated skin was stained with the anti–E-selectin mAb EL-246 (*arrow*, intense luminal staining). Approximate magnification is 200.

and similar to what has been described for the glycoprotein receptor for P-selectin (35). However, only the 250-kD molecule, and not the slower mobility digested product (300 kD), was able to interact with E-selectin. These results show that there are only a limited number of protein species (even under these low stringency conditions) that can be bound by E-selectin. Furthermore, the 250-kD molecule's specificity for E-selectin is EDTA and neuraminidase sensitive, the same as γ/δ T cell binding to the E-selectin–L cells.

We then tested for a similar E-selectin binding molecule in lysates generated from two tumor cell lines (L1.2 and L cells). L1.2 cells (mouse pre-B tumor cell line) demonstrated a low affinity capacity to interact with the E-selectin–L cells (data not shown). 5×10^8 L1.2 cells were lysed and run over the preclearing and E-selectin columns as described above for the γ/δ T cells. The EDTA elution fractions off the EL-81–E-selectin column were consolidated, and the same was done with elution fractions from a γ/δ T cell lysate of equivalent cell numbers. An equal sample volume (reflecting material from the same number of cells) from each of the two preparations was examined by silver stain analysis. Interestingly, a 250-kD molecule was isolated from the L1.2 cells; however, the expression level was far less than on γ/δ T cells (Fig. 3 *C*), which correlated with the L1.2 cell's low E-selectin binding capacity ($\sim$1:20 the capacity of γ/δ T cells). Similar analysis was done with L cells, a mouse nonhematopoietic tumor cell line, in which the 250-kD molecule was not detected (Fig. 3 *C*). The 300- and 200-kD proteins detected in the L cell elution fractions (Fig. 3 *C*) were eluted off the preclearing column as well, indicating they were interacting with EL-81 and/or the supporting matrix, but not E-selectin (data not shown).

Upregulation of E-Selectin in Bovine Skin Is Associated with Increased γ/δ T Cell Localization. Animals of $\sim$8 wk of age were stimulated by an intradermal injection of 5–10 μg TNF-α, a known inducer of E-selectin expression (5, 45), to examine γ/δ T cell recruitment. Sections cut from control and stimulated skin samples were stained by immunoperoxidase using the γ/δ T cell–specific mAb IL-A29, the α/β T cell–specific mAb CC42, or the anti-E-selectin mAb EL-246. Fig. 4 *A* shows γ/δ T cells from a representative region of control skin. Fig. 4 *B* shows γ/δ T cells from a representative region of TNF-α-stimulated skin (note the perivascular accumulation of γ/δ T cells). The same TNF-α-stimulated skin, stained with the anti–E-selectin mAb EL-246, demonstrated intense luminal expression of E-selectin on the dermal blood vessels (Fig. 4 *C*). Using serial cut sections, we compared the ratio of γ/δ T cells to α/β T cells actively bound to the luminal surface of venules in TNF-α-inflamed skin. This analysis allowed for the quantitation of newly recruited lymphocytes versus cells, both γ/δ and α/β T cells, that were already in the tissue before induction of inflammation. The γ/δ to α/β T cell ratio in peripheral blood was $\sim$0.7:1, however, the ratio found in inflamed dermal venules was 3.7:1 (determined from analysis of >200 cells). These results suggest that there is a correlation between the upregulation of E-selectin expression and increased γ/δ T cell accumulation.

Discussion

E-selectin was originally defined as an inducible endothelial cell adhesion protein for myeloid cells (5, 45) and is believed to be important in regulating acute inflammatory responses. Recent studies have expanded the role of E-selectin to serving as a putative vascular addressin in certain chronic inflammatory processes for a subset of human memory T cells (8–11). We add to these studies by demonstrating the capacity of ruminant γ/δ T cells from neonatal and mature animals to bind E-selectin. This interaction is supported by our in vivo studies in which induction of E-selectin expression by TNF-α in bovine skin is associated with an influx of γ/δ T cells at the same site. Our analyses are directly relevant to the human, since a significant percentage of human γ/δ T cells express the HECA-452 epitope, which Berg et al. and Picker et al. (2, 8, 10–12, and see below) have shown correlates precisely with the ability of lymphocytes to bind E-selectin, and we have found that a small percentage of human lymphocytes that bind E-selectin are stained by anti-γ/δ TCR mAbs (data not shown). Thus, E-selectin has the potential of regulating many diverse leukocyte–endothelial cell interactions that occur in different inflammatory settings.

Norton et al. (46, 47) have suggested that particular tissues may constitutively express low levels of E-selectin on certain venules. If true, E-selectin may be involved in leukocyte extravasation in the absence of overt inflammation. This hypothesis is directly relevant to γ/δ T cells. The tissues that have been shown to constitutively express low levels of E-selectin are the skin and gut: both are sites of γ/δ T cell localization (13, 14). Preliminary analysis suggests that γ/δ T cells bind E-selectin more avidly than other leukocytes (data not shown). If true, low levels of E-selectin on certain venules may preferentially recruit γ/δ T cells and account for their localization in uninflamed skin and gut. Indeed, E-selectin may serve the same role in certain extralymphoid tissues as the vascular addressins play in lymph nodes and Peyer's patches.

In this study, we provide a preliminary characterization of the adhesive mechanism used by γ/δ T cells to bind E-selectin. A previously described component of leukocyte receptors for E-selectin is sialylated carbohydrates. sLex on neutrophils (36, 39) and the HECA-452 epitope (CLA antigen) on human memory lymphocytes (8, 10) are examples of these carbohydrate structures. Sialylated carbohydrates appear important in the binding of γ/δ T cells to E-selectin as well, since neuraminidase treatment of the lymphocytes inhibits the interaction. However the CSLEX and HECA-452 mAbs do not recognize γ/δ T cells, suggesting that additional biological carbohydrate structures, antigenically different from what has been described on myeloid cells and CLA$^+$ lymphocytes, are recognized by E-selectin.

The carbohydrate ligands are only a partial representation of the entire receptor for selectins. It is likely that the biological receptors for the selectins would also be composed of lipid or protein. Importantly, both P- and L-selectin have been shown to bind specific cell surface glycoproteins with high affinity (35, 48). The complete significance of this protein component has not been determined. Protein may provide

for a "scaffolding" in which carbohydrate structures are presented to the selectins (48). However, it is possible that certain regions of the protein backbone may participate in a protein–protein interaction with the selectins, in addition to the carbohydrate–lectin interaction.

Studies addressing the nature of the biological receptor on leukocytes for adhesion to E-selectin vary considerably in their findings. sLex and the HECA-452 antigen are found on many different glycoproteins and glycolipids on the leukocyte surface, suggesting that a large number of molecules could potentially serve as receptors (42). However, this does not appear to be the case. Activation of neutrophils actually increases sLex expression, but binding to E-selectin is greatly diminished (28, 42, 49). Thus, there appears to be a restriction in the types of molecules on the leukocyte surface that can bind E-selectin.

Putative glycoprotein receptors for E-selectin have been described on neutrophils. For example, L-selectin may preferentially present sLex on neutrophils to E-selectin. Anti–L-selectin mAbs block neutrophil adhesion to E-selectin cDNA transfectants to the same extent as anti–E-selectin mAbs, suggesting that E- and L-selectin can serve as receptor–ligand pairs (28), Picker et al. (42) extended these observations and found that L-selectin is decorated by sLex, and its preferential localization at the tips of the microvilli and ruffles of the neutrophil cell surface membrane may account for its predominant role in presenting carbohydrates to E-selectin. A recent study by Vestweber et al. (50) has described an additional glycoprotein (160 kD, reducing conditions) that can be isolated from detergent lysates of mouse neutrophils by an E-selectin affinity column. However, an analogous role for L-selectin or any other single glycoprotein in mediating lymphocyte adhesion to E-selectin has not been shown.

We show that protein is a major component of the γ/δ T cell receptor for E-selectin. Treatment of γ/δ T cells with chymotrypsin or trypsin (to a lesser degree) blocks the binding interaction. L-Selectin, CD44, and CD18, on γ/δ T cells do not appear to represent the surface receptor. In particular, three different approaches that block L-selectin function had no significant effect on γ/δ T cell/E-selectin adhesion.

To address whether single or multiple cell surface glycoproteins can potentially serve as E-selectin ligands, we examined NP-40 solubilized membrane molecules from γ/δ T cells by an E-selectin affinity column. The affinity isolation approach used here purified a single 250-kD glycoprotein that required sialic acid and divalent cations for an interaction with E-selectin. A similar size molecule was not found in a lysate of a nonhematopoietic tumor cell line (L cells). Lysates of a lymphoid tumor cell line (L1.2) did contain the 250-kD molecule; however, the apparent expression level was much less than that from an equal number of γ/δ T cells. The low expression of the putative E-selectin receptor by the L1.2 cells was not surprising for two reasons: low numbers of L1.2 cells are capable of binding E-selectin–L cells under rotation, and a recent study by Vestweber et al. (50) using an E-selectin–IgG fusion protein has identified a predominant 160-kD molecule and a minor 250-kD molecule from detergent lysates of mouse leukocytes isolated from bone marrow. The 160-kD molecule appears to be myeloid specific, but the 250-kD molecule may have been derived from the minor bone marrow lymphocyte population. In consideration of the proposed glycoprotein receptors for L- and P-selectin, the γ/δ T cell receptor for E-selectin may represent an additional member of a potential new family of highly glycosylated surface proteins that serve as high affinity ligands for selectins.

It was beyond the intent of this study to provide a detailed biochemical characterization of the 250-kD molecule. Such analysis will include the generation of antibodies to determine the biological significance of this molecule. Our affinity isolation technique allows for the purification of protein in the microgram range, thus, providing a significant quantity for immunizations. Antibodies will be necessary to definitively establish a functional role for the 250-kD molecule at the level of the in vitro binding assay and eventually in vivo. Interestingly, the molecule defined here is related in several ways to two other recently described molecules: the proposed receptor for P-selectin isolated from the surface of neutrophils, which is ~250 kD (under nonreducing conditions) (35); and the WC1 antigens (also referred to as T19) (14, 19, 51, 52), a recently cloned, lineage-specific, γ/δ T cell surface glycoprotein whose function is unknown (20). Because of the WC1 antigens' selective expression, it has been proposed to be an epithelial-associated tissue homing receptor for γ/δ T cells (22). Immunoprecipitation of the WC1 antigen reveals a predominant molecular species of 165 kD (nonreducing conditions) (19). Some anti-WC1 antibodies immunoprecipitate additional minor species with molecular masses above and below the 165-kD antigen (22). It is possible the 250-kD glycoprotein defined here represents one of these minor forms of WC1. Our testing of this hypothesis to date has been incomplete; however, anti-T19 mAbs do not block γ/δ T cell adhesion to E-selectin. To confirm the similarity of these three proteins, antibodies generated against our 250-kD molecule will be tested for antigenic crossreactivity towards the receptor for P-selectin and the WC1 antigens.

In conclusion, our results provide the first demonstration that lymphocytes in an animal model bind E-selectin. Ruminant γ/δ T cells adhere to E-selectin with high avidity and may use a previously undescribed 250-kD glycoprotein as their receptor. To date, there has been no in vivo evidence that blocking E-selectin alters lymphocyte extravasation. This is because virtually all studies of the interaction between lymphocytes and E-selectin have been done in the human where reagents and assays are readily available, but homing experiments cannot be done. Defining lymphocyte–E-selectin interactions in ruminants provides a significant advancement because of the potential for in vivo analysis. Defining the γ/δ T cell system in ruminants is particularly advantageous because these cells comprise 40–60% of the circulating T cell pool (14), and it can be expected that if their circulatory pathways are blocked a biological effect can be readily measured.

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